1724–1730 *Nucleic Acids Research, 1998, Vol. 26, No. 7*

Signal-dependent degradation of IκBα is mediated by an inducible destruction box that can be transferred to NF-κB, Bcl-3 or p53

F. Gregory Wulczyn+, Daniel Krappmann and Claus Scheidereit*

Max-Delbrück-Center for Molecular Medicine, MDC, Robert-Rössle-Straße 10, 13122 Berlin, Germany

Received November 28, 1997; Revised and Accepted February 6, 1998

ABSTRACT

Activation of the transcription factor NF-κB in response to a variety of stimuli is governed by the signal-induced proteolytic degradation of NF-κB inhibitor proteins, the IκBs. We have investigated the sequence requirements for signal-induced IκBα phosphorylation and proteolysis by generating chimeric proteins containing discrete sub-regions of IκBα fused to the IκBα homologue Bcl-3, the transcription factor NF-κB1/p50 and the tumour suppressor protein p53. Using this approach we show that the N-terminal signal response domain (SRD) of IκBα directs their signal-dependent phosphorylation and degradation when transferred to heterologous proteins. The C-terminal PEST sequence from IκBα was not essential for induced proteolysis of the chimeric proteins. A deletion analysis conducted on the SRD identified a 25 amino acid sub-domain of IκBα that is necessary and sufficient for the degradative response *in vivo* and for recognition by TNFα-dependent IκBα kinase *in vitro*. The results obtained should prove instrumental in the further characterization of IκB-specific kinases, as well as the E2 and E3 enzymes responsible for IκBα ubiquitination. Furthermore, they suggest a novel strategy for generating conditional mutants, by targetting heterologous proteins for transient elimination by the IκBα pathway.

INTRODUCTION

A hallmark of the regulation of the ubiquitous transcription factor NF-κB is its activation by release from associated inhibitory molecules, the IκBs. To date five vertebrate NF-κB/Rel proteins are known: p50, p52, p65 (RelA), c-Rel and RelB. Active nuclear NF-κB is dimeric and the NF-κB subunits form hetero- and homodimeric complexes with discrete functional activity and cell type specificity. These dimers are harnessed to signal transduction pathways by their interaction with the cytoplasmic IκB molecules IκBα, IκBβ, IκBε, p105 and p100, as well as the predominantly nuclear IκB homologue Bcl-3 (see references 1–4 for recent reviews). All IκB molecules share a characteristic conserved domain, the ankyrin repeat cluster, which mediates IκB association with NF-κB dimers. This association can prevent recognition of the NF-κB nuclear localization signal, resulting in cytoplasmic retention of NF-κB. With the exception of tyrosine phosphatase (5), NF-κB inducing agents act by targetting IκBs for site-specific serine phosphorylation and subsequent proteolytic degradation by the ubiquitin-mediated proteasome pathway (reviewed in 2,6).

The activation pathway has been most intensively investigated in the context of the NF-κB p50/p65 heterodimer in complex with IκBα. It has been established that a large number of inducing agents, including TNF-α, LPS, IL-1, PMA and the HTLV-1 Tax protein, act by a common mechanism involving phosphorylation of IκBα at Ser32 and Ser36. Mutation of these residues prevents both signal-induced IκBα phosphorylation (7–10) and subsequent poly-ubiquitination (11). The major sites for ubiquitin attachment have been mapped to Lys21 and Lys22 (12–14) and it has been shown that the ubiquitinated substrate is degraded by the proteasome (11,15,16). A multisubunit IκBα kinase complex of ~700 kDa has been characterized which phosphorylates IκBα at Ser32 and Ser36 (17). Intriguingly, the activity of this kinase itself or of another component in the kinase complex may require ubiquitination (17).

The critical serine and lysine residues governing IκBα degradation reside in the N-terminal portion of IκBα, which has been termed the signal response domain (SRD). The C-terminus of IκBα contains a sequence rich in acidic residues (serines, threonines and prolines) that meets the criteria established for the PEST motif (18). Originally identified as a motif correlating with protein instability, PEST sequences have been shown to control regulated proteolysis of the yeast G_1 cyclins CLN2 and CLN3 (reviewed in 19,20) and of cyclin E in mammals (21,22). The role of the IκBα pest domain is controversial; a number of studies have suggested that the C-terminal PEST sequences contribute to induced degradation (8,10,23), while other studies have concluded that the PEST sequence is not required (24,25). In contrast, it has been clearly established that the C-terminal domain, including the PEST motif, cooperates with the ankyrin repeat domain in determining the affinity of the IκBα–p65 interaction (26–28). The possible significance of NF-κB–IκB complex formation for degradation efficiency has not been directly addressed and might account for some of the experimental variability observed.

Although previous studies have identified the serine residues that are phosphorylated and lysine residues that are ubiquitinated during signal-induced degradation of IκBα, less is known about the substrate requirements for each of these reactions. An important step in understanding how the specificity and efficiency of the IκBα pathway is achieved is to determine the molecular

*To whom correspondence should be addressed. Tel: +49 30 9406 3816; Fax: +49 30 9406 3866; Email: scheidereit@mdc-berlin.de

+Present address: Department of Pathology, Anatomy and Cell Biology, Jefferson Medical College, 1020 Locust Street, Philadelphia, PA 19107, USA

recognition elements directing each step. These include recognition by the IκBα kinase complex, ubiquitin conjugating enzymes and by the proteasome. We have addressed these issues by constructing a series of chimeric proteins in which the control domains of IκBα have been fused to the non-responsive IκBα homologue Bcl-3, to the unrelated NF-κB p50 subunit or to the tumour suppressor protein p53. After completion of this work, during preparation of the manuscript, Brown *et al*. published the results of a very similar experimental strategy (29; see Discussion). We report here the identification of N-terminal IκBα sequences that could confer signal-induced phosphorylation both *in vivo* and *in vitro* and signal-induced degradation *in vivo* to each of the heterologous proteins tested. This sequence contains previously mapped serine and lysine residues required for signal-induced phosphorylation and ubiquitination respectively. In contrast, the C-terminal PEST sequence of IκBα did not act autonomously to induce degradation of the chimeric constructs tested, although it strongly enhanced degradation of IκBα. The delineated N-terminal IκBα sequence represents a novel type of transferable 'destruction box' and is the first such sequence shown to be regulated by defined signal transduction pathways.

MATERIALS AND METHODS

Plasmid construction

pCBcl3 contains the full-length open reading frame of human Bcl-3 in pCDNA3 (Invitrogen). The construct is N-terminally tagged with an epitope from T7 gene *10* and a hexa-histidine repeat, identical to that of the pCIκBα expression construct (28). IκBαΔN (amino acids 71–317) and IκBαΔC (amino acids 1–279) have been previously described (28). To generate N-terminal fusion proteins to the ankyrin repeat domain of Bcl-3, Bcl-3 sequences between position 95 and 226 were replaced by an *Xba*I linker after destruction of the *Xba*I site in the vector. Sequences between the *Xba*I linker and an upstream *Hin*dIII site in the vector were replaced by PCR fragments generated from pCIκBα. Two clockwise primers were used, one vector derived (T7 promoter), the second with sequence GGGGGATCCTGAAGAAGGAGCGGCTAC. These primers were combined with three counterclockwise primers (GGTGTCTAGATGCTTCCAGGCTCCGAGC, CACCTCTAGAGGCTCGAGGCGGATCTCCTG and CTCCCTAGAATCTGCTCGTACTCCTCGTC) to generate fragments encoding IκBα amino acid sequences 1–67, 1–55, 1–44, 20–67, 20–55 and 20–44. To exchange C-terminal sequences PCR fragments were generated with a *Kpn*I site replacing bases 918–923 of Bcl-3 or 818–823 of IκBα at a homologous position at the junction of ankyrin repeats 5 and 6 in each protein. IkB::p53 and IkB::p50 fusion constructs were generated by PCR. The fidelity of PCR synthesis was confirmed by sequencing plasmid constructs. Further details regarding the cloning is available upon request. CMVp53 was provided by M.Strauss, IκBα(32A36A) was a gift of A.Israel (10).

The amino acid sequences contained in each of the chimeric constructs is as follows: chimera A, Bcl-3 amino acids 1–292 and IκBα amino acids 244–317; chimera B, IκBα amino acids 1–242 and Bcl-3 amino acids 294–447; chimera C, IκBα amino acids 1–67, Bcl-3 amino acids 63–447, IκBα amino acids 279–317; chimera D, N-terminal IκBα sequences as indicated in the text, fused to Bcl-3 amino acids 63–292 and IκBα amino acids 244–317; chimera E, N-terminal IκBα sequences as indicated in the text, fused to Bcl-3 amino acids 63–292; IkB::p50, IκBα amino acids 1–67, fused to p50 amino acids 1–376; IkB::p53, IκBα amino acids 1–67, fused to full-length human p53.

Cell culture

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin/streptomycin and 1 mM sodium pyruvate. Transient transfections were carried out with lipofectamine (Gibco BRL) following the manufacturer's protocols and harvested 16 or 36 h after transfection. Total DNA amounts, including carrier DNA, yielding optimal transfection efficiency were determined; the lowest amount of each expression plasmid allowing consistent detection in Western blots was then used. Stable transfectants were pooled survivors of selection with 600 μg/ml G418 (Gibco BRL), with the exception of IκBα, IκBαΔN, IκBαΔC and Bcl-3, which were clonal lines. HeLa cells were stimulated with 20 ng/ml tumour necrosis factor α (TNF-α; Biomol) for 15 min or with 0.5 μM okadaic acid (OA; Calbiochem) for 90 min. Cells were pretreated for 15 min with 50 μg/ml *N*-acetyl-leucyl-leucyl-norleucinal (ALLN; calpain I inhibitor, Boehringer) to inhibit proteasome activity.

Electrophoretic mobility shift assay (EMSA) and Western blotting

For stimulation with TNF-α cells were washed twice in PBS, lysis buffer (20 mM HEPES, pH 7.9, 350 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM $MgCl_2$, 20% glycerol, 1% Nonident P-40, 1 μg/ml Pefabloc, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 mM NaF, 8 mM β-glycerophosphate, 1 mM DTT) was added and the cells frozen in liquid nitrogen. The cell lysate was allowed to thaw on ice, scraped and transferred to microcentrifuge tubes. The supernatant formed by 10 min centrifugation at 14 000 r.p.m. was used for further analysis. Stimulation with OA frequently resulted in cell detachment. The culture supernatant was collected, harvested as above and combined with the lysate from the adherent cell fraction. Preincubation with ALLN had no effect on cell detachment. Protein concentration was determined using the BCA protein assay (Pierce).

Electrophoretic mobility shift assays (EMSA) were performed as described previously (30). Western blots were analysed by chemiluminescense following the manufacturer's recommendations (Tropix or New England Biolabs). Chimeric molecules were detected with antibodies directed against the IκBα N- or C-terminus obtained from Santa Cruz Biotechnology (C-15 and C-21). Other antibodies were obtained from Rockland (anti-p50) and Calbiochem (p53 mAb-2); Bcl-3 was detected with a peptide antibody raised against the 15 N-terminal amino acids of Bcl-3.

In vitro phosphorylation

^{35}S-Labelled proteins used as substrates for phosphorylation reactions were synthesized using a coupled *in vitro* transcription/translation reaction (Promega). Extract preparation and reaction conditions for *in vitro* phosphorylation are described in Lee *et al*. (31).

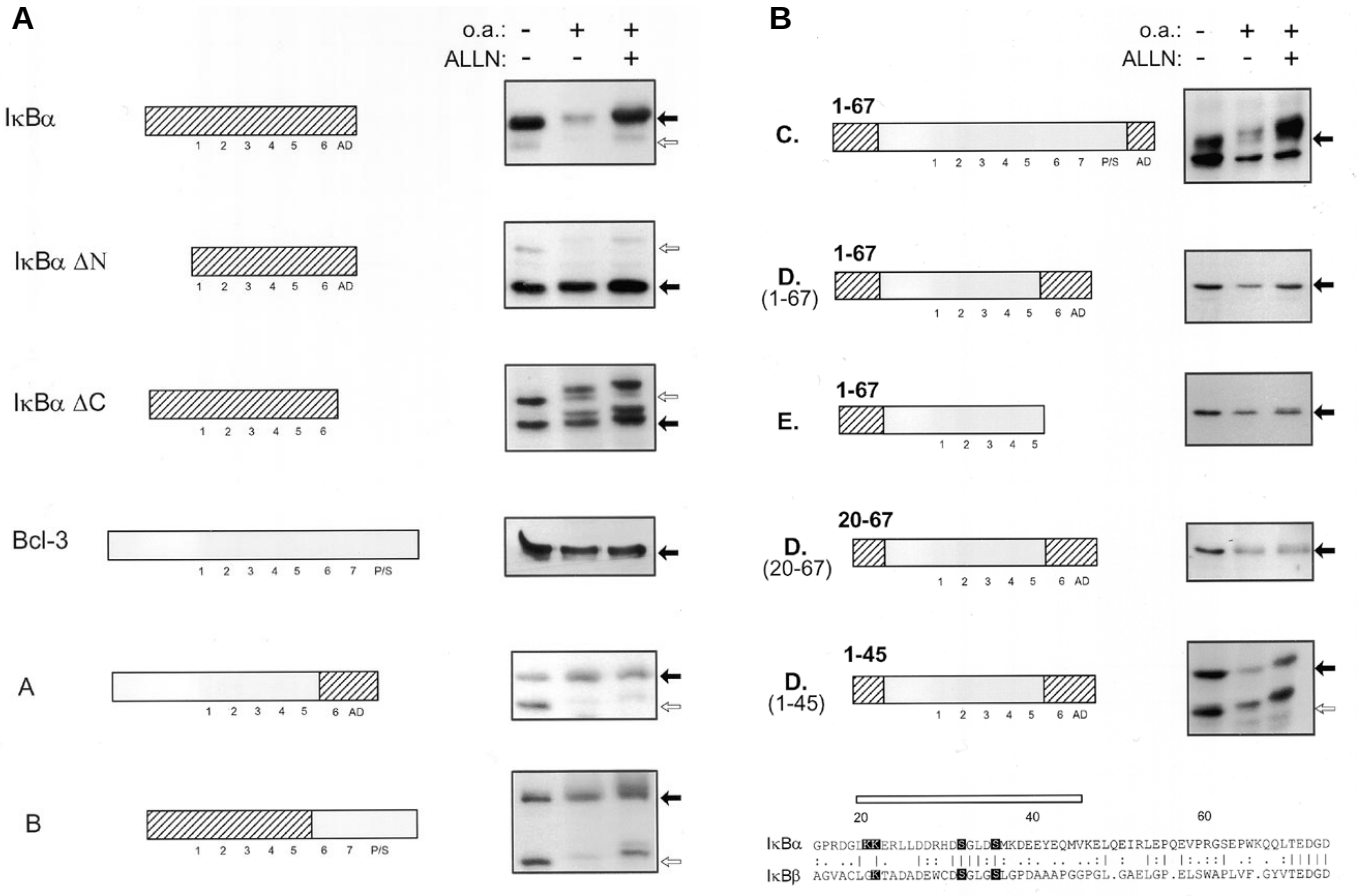

Figure 1. IκBα domains required for response to okadaic acid *in vivo*. (**A** and **B**) The constructs referred to in the text are schematically illustrated to the left of each panel. IκBα-derived sequences are designated by striped boxes, Bcl-3 sequences are in grey. Numbers refer to the position of ankyrin repeats 1–6 in the case of IκBα and 1–7 in the case of Bcl-3. P/S refers to the proline + serine-rich region in the C-terminus of Bcl-3. AD refers to the acidic domain in IκBα. Corresponding Western blots are shown in the panels to the right of each construct. Extracts were prepared from unstimulated control cells, from cells stimulated with okadaic acid (o.a.) and from cells pretreated with ALLN prior to okadaic acid treatment as indicated at the top. The filled arrow designates the product of the transfected construct, the open arrow designates endogenous IκBα, when appropriate. In all other cases the endogenous IκBα response was confirmed in a separate gel using an IκBα-specific antibody. Migration of construct C is heterogeneous, reflecting constitutive hyperphosphorylation of the C-terminal domains. Equal amounts of protein were applied to each lane, as determined using the Micro-BCA Protein Assay Kit (Pierce). Equilavent loading was further verified using the signals from cellular proteins that cross-react with the biotinylated second antibody used in the Western procedure (data not shown). At the bottom of (B) a sequence alignment of the N-terminal region of IκBα and IκBβ is presented. Ser32, Ser36, Lys 21 and Lys22, referred to in the text, are highlighted. The open bar marks the position of the destruction box defined in this study.

RESULTS

Induced phosphorylation and degradation of chimeric Bcl-3–IκBα proteins largely depends on 25 N-terminal residues of IκBα

To investigate signal response elements in IκBα we performed a ‘domain swap’ experiment in which N-and C-terminal domains of IκBα were exchanged with their counterparts in the IκB homologue Bcl-3. In the first series of experiments stably transfected HeLa cells were induced with OA, a potent inducer of NF-κB (Fig. 1). In cells expressing epitope-tagged wild-type IκBα (Fig. 1A, top, filled arrow) the transfected construct was efficiently degraded after administration of OA, in parallel with endogenous IκBα (open arrow). Pretreatment of cells with ALLN, a protease inhibitor active against the proteasome, efficiently inhibited IκBα degradation and led to the appearance of an IκBα form of reduced mobility. It has been previously established that this form represents a phosphorylated species, an intermediate in the signal-induced phosphorylation of IκBα (8,11). Removal of the first 70 amino acids of IκBα, corresponding to the SRD (Fig. 1A, IκBαΔN), rendered the molecule resistant to both OA-induced phosphorylation and degradation. Similar observations, consistent with the requirement for Ser32, Ser36, Lys20 and Lys21 within the SRD for signal-dependent phosphorylation and degradation, have been made by several laboratories (6). Deletion of the C-terminal sequences of IκBα from amino acid 280 (Fig. 1A, IκBαΔC) reduced the degradative response to OA, however, comparing the steady-state amounts detected after OA treatment with or without ALLN shows that some degradation did occur (Fig. 1A, IκBαΔC, compare lanes 2 and 3). In contrast, Bcl-3 did not respond to OA. Equivalent results were obtained using the same constructs in transient transfection assays as detailed in Materials and Methods (data not shown). To test the relative contributions of the IκBα N- and C-terminal domains to the OA response in a heterologous protein both domains were exchanged with the corresponding sequences from Bcl-3. Transfer of the complete C-terminal domain of IκBα, including the sixth ankyrin repeat and the PEST

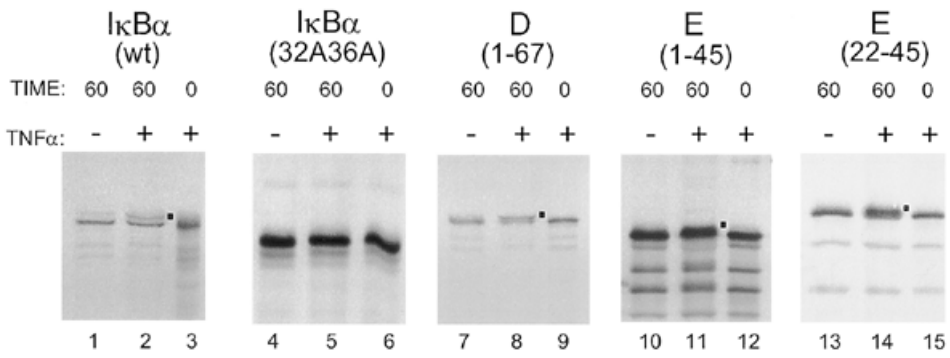

Figure 2. *In vitro* analysis of TNF-α-dependent SRD phosphorylation. Substrate proteins were generated by transcription and translation *in vitro*. Reactions contained extracts from either unstimulated or TNF-α-stimulated HeLa cells as indicated. Parallel reactions were terminated either immediately, by addition of SDS gel loading buffer, as a control (0 min incubation) or after a 60 min incubation as indicated. Substrate proteins are indicated above each panel and are described in the text and in Figure 1. Bands arising from TNF-α-dependent phosphorylation are marked by dots.

motif, to Bcl-3 did not confer any responsiveness to the resultant chimeric protein (Fig. 1A, chimera A). Chimera B is the complementary construct in which the IκBα C-terminus is replaced with the C-terminus from Bcl-3. This chimera was partially degraded after OA administration and slower migrating phosphorylated forms accumulated after treatment with OA + ALLN. Although the Bcl-3 C-terminus could not completely complement deletion of the IκBα C-terminus, it can be concluded that the primary determinants mediating inducible degradation of the chimeric proteins are contained in the SRD and possibly in the repeat cluster of IκBα.

To further dissect the responsive domains a series of chimeric proteins was generated containing the SRD from IκBα linked to Bcl-3 (Fig. 1B, constructs C–E). In addition to the SRD, construct C contains the IκBα PEST sequence fused to the complete C-terminal sequence of Bcl-3. In constructs D and E the C-terminus of Bcl-3 has been either replaced with the C-terminus of IκBα (construct D) or deleted (construct E). Each of these chimeric proteins was readily degraded after OA treatment and accumulated as hyperphosphorylated forms after treatment with OA + ALLN (Fig. 1B). The panels presented in Figure 1B are representative of three independent transient transfection experiments comparing constructs D and E. In each experiment degradation of construct E was at least as efficient as construct D, an observation that was further confirmed using populations of cells stably expressing each construct (data not shown). We conclude that the IκBα PEST sequences are not required for inducible degradation of these chimeric constructs. The primary sequence determinants recognized by both the IκBα kinase and the ubiquitin conjugating enzymes are contained within the N-terminus of IκBα (see Discussion).

The extent of the SRD within the IκBα N-terminus was further defined in a deletion analysis. These experiments were facilitated by the C-terminal IκBα epitope present on construct D, however, the same deletion end-points introduced into construct E gave comparable results (data not shown). Amino acids 1–19 and 46–67 were dispensable for the response to OA (Fig. 1B, constructs D1–45 and D20–67). These data thus suggest that residues 20–45 of IκBα, containing Ser32, Ser36, Lys21 and Lys22, represent an inducible destruction box and are sufficient to confer inducible degradation. It is important to point out that we never observed inducible phosphorylation in the absence of degradation. This implies that the recognition determinants for the IκBα kinase and the IκBα-specific ubiquitination enzymes must overlap. The N-terminus of this sequence is demarcated by Lys21 and Lys22; further deletion would be predicted to affect inducible ubiquitination. At the C-terminus of the SRD a gradual loss of responsiveness was observed when comparing the activity of constructs ending at positions 67, 55 and 45 in transient transfection assays (data not shown). Cells stably expressing D1–45, however, responded to the same degree as D1–67.

The destruction box of IκBα is inducibly phosphorylated *in vitro*

Optimal *in vivo* phosphorylation and degradation of the chimeric proteins were observed with OA, a phosphatase inhibitor that activates the IκBα degradation pathway persistently. We next chose an *in vitro* phosphorylation assay using the chimeric molecules as substrate, to confirm that the *in vivo* phosphorylation events can be attributed to cytokine-inducible IκBα kinase. We made use of a recently developed *in vitro* assay that faithfully reproduces TNFα-dependent IκBα kinase activity (31). Cytosolic extracts prepared by a rapid lysis procedure from unstimulated or TNF-α-induced HeLa cells were incubated with ^{35}S-labelled *in vitro* translated substrate proteins in the presence of an ATP regenerating system (Fig. 2).

Full-length IκBα was phosphorylated in extracts of TNF-α-stimulated cells but not in extracts of unstimulated cells (lanes 1–3), as shown by Lee *et al*. (31). The TNF-α-induced IκBα kinase activity was specific for Ser32 and Ser36 of IκBα, since the Ala32/Ala36 double mutant was not phosphorylated (second panel, lanes 1–3). Chimeras D(1–67), E(1–45) and E(20–45) were all phosphorylated in extracts of TNF-α-stimulated cells, but not in control extracts (Fig. 2), with an efficiency comparable with that of native IκBα. Thus all chimeric proteins containing the IκBα destruction box were phosphorylated in a TNF-α-dependent fashion. There are no serine or threonine residues in the destruction box (amino acids 20–45) other than Ser32 and Ser36. Since the IκBα Ser32/Ser36 double mutant was not phosphorylated, we conclude that the latter residues were modified in the chimeric proteins.

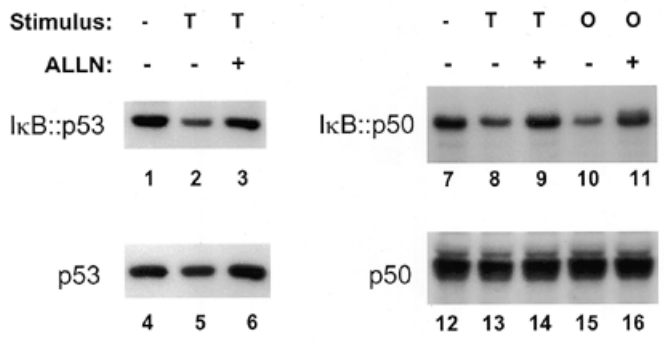

Figure 3. Western blot analysis of IkB::p53 and IkB::p50 constructs. On the left are results obtained with a SRD (amino acids 1–67)–p53 fusion protein (upper panel) and native p53 (lower panel). Both proteins were detected with p53 mAb2. Results obtained with a SRD (amino acids 1–67)–p50 fusion construct (upper panel) are shown on the right. Endogenous p50 from the same cells was detected with a p50-specific antibody (lower panel). Peptide competition experiments verified the position of the p50 signal; it co-migrates with a weaker diffuse non-specific signal (data not shown). The response of endogenous IκBα was confirmed after stripping the gels and reprobing with IκBα-specific antibody (data not shown).

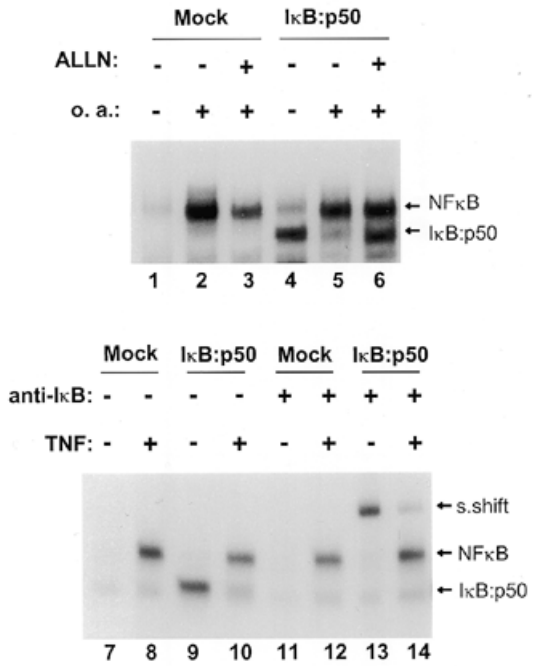

Figure 4. Functional analysis of the IκBα–p50 response. DNA binding assays were performed using the NF-κB site from the H2K enhancer as probe. Only the specific DNA–protein complexes are shown. (Top) Proteolytic degradation of IκBα–p50 in response to okadaic acid. Extracts were prepared from mock-transfected cells (lanes 1–3) and from cells expressing the IkB::p50 fusion construct (lanes 4–6). Extracts applied to the gel were from unstimulated cells (lanes 1 and 4), from cells stimulated with okadaic acid (lanes 2 and 5) and from cells stimulated with okadaic acid after pretreatment with the protease inhibitor ALLN (lanes 3 and 6). (Lower) Analysis of DNA binding complexes with anti-IκBα antibody. Extracts were prepared from mock-transfected cells (lanes 7–8 and 11–12) and from cells expressing the IkB::p50 fusion construct (lanes 9–10 and 13–14). Extracts applied to the gel were from unstimulated cells (odd numbered lanes) and from cells stimulated with TNF-α (even numbered lanes). Extracts from lanes 7–10 were challenged with an antibody directed against the IκBα SRD carried by the fusion protein. Migration of endogenous NF-κB, IκBα–p50 and antibody–IκBα–p50 complexes are denoted by arrows.

Inducible proteolysis of tumour suppressor protein p53 and NF-κB subunit p50 after fusion to the IκBα signal response domain

To test whether the IκBα destruction box requires the presence of an ankyrin repeat domain or if it can function more generally, further chimeric proteins were generated. The IκBα destruction box was fused to the N-termini of both the tumour suppressor protein p53 and the p50 subunit of transcription factor NF-κB. In a transient transfection assay the IκBα–p53 fusion protein was sensitive to TNF-α-mediated proteolysis (Fig. 3, lanes 1–3). Native p53 displayed increased accumulation in the presence of ALLN, presumably due to a reduction in its high rate of turnover in HeLa cells, but p53 degradation was not appreciably increased in response to TNF-α (lanes 4–6). In a similar fashion, the presence of the destruction box caused efficient degradation of chimeric p50 after stimulation with either TNF-α or OA (Fig. 3, lane 7 compared with 8 and 10) and degradation was blocked in the presence of ALLN (lanes 9 and 11). Stimulation with OA in the presence of ALLN led to accumulation of phosphorylated chimeric p50 (lane 11). As expected, endogenous p50 was not responsive to TNF-α or OA stimulation (lanes 12–16).

To follow the functional consequence of chimeric p50 degradation for endogenous NF-κB complexes mobility shift assays were performed (Fig. 4). In mock-transfected cells OA treatment led to the expected induction of endogenous NF-κB activity; stabilization of IκBα in the presence of ALLN strongly reduced NF-κB activation (lanes 1–3). Transfected chimeric p50 migrated as a unique DNA complex (cf. lanes 1 and 4) that was strongly diminished after OA treatment; activation of endogenous NF-κB was unaffected (lane 5). ALLN pretreatment blocked OA-induced breakdown of chimeric p50 (lane 6). As with OA, TNF-α treatment caused disappearance of DNA-bound chimeric p50 (lanes 9 and 10), whereas DNA binding activity of endogenous NF-κB was induced by TNF-α (lanes 7–10). DNA-bound chimeric p50, but not endogenous NF-κB, was supershifted with an antibody directed against residues 6–20 of IκBα (lanes 7–14). The supershifted complex was also strongly reduced after TNF-α treatment, as a consequence of induced proteolysis of chimeric p50 (lanes 13 and 14).

The findings with chimeric p50 and p53 are thus in accordance with the conclusions drawn from the chimeric IκBα–Bcl-3 proteins. In each case the N-terminal destruction box of IκBα was necessary and sufficient for TNF-α- or OA-induced degradation of the entire protein chain in the absence of further sequences from IκBα

DISCUSSION

A key event in the activation of NF-κB by a large number of physiological and pharmacological agents is regulated proteolytic degradation of IκB molecules. In studies by a number of laboratories using both biochemical and genetic approaches it has been established that signal-induced phosphorylation of Ser32 and Ser36 triggers ubiquitination of IκBα, predominantly at Lys21 and Lys22, leading to proteolytic degradation by the 26S proteasome (reviewed in 3,6). Nevertheless, comparatively little is known about the structural requirements and the specificities governing recognition of IκB proteins by modifying enzymes. Although Ser32 and Ser36 have been mapped as phosphorylation

sites essential for IκBα degradation, it is unclear whether these residues are in fact sufficient or whether they serve as primary phosphorylation sites, modification of which would induce phosphorylation of other secondary but equally essential sites. Furthermore, different conclusions have been drawn about a requirement for the PEST sequence or for the ankyrin repeat domain for IκB degradation. It is also a matter of debate whether rapid signal-induced IκBα breakdown requires that IκBα be bound to NF-κB.

To address these questions we have designed a series of chimeric proteins to identify sequences in IκBα controlling phosphorylation, ubiquitination and proteolytic degradation. As a first step we constructed IκBα–Bcl-3 fusion proteins to determine if sub-regions of IκBα can direct induced degradation in a structurally related context, e.g. when embedded in a protein with ankyrin repeats and the potential ability to bind NF-κB. Signal-induced phosphorylation and degradation could be transferred to Bcl-3 by the N-terminal domain of IκBα. We have subsequently fused this IκBα destruction box to p50 and to p53 and found that both chimeras were readily degraded in response to either TNF-α or OA. We have thus identified an inducible and transferable destruction box in IκBα that mediates proteolysis of a fusion protein, no matter whether this is the ankyrin repeat domain of IκBα or of Bcl-3 or the transcription factors NF-κB1/p50 or p53. The destruction box was also shown to be the target of OA-induced phosphorylation *in vivo* and of TNF-α-dependent phosphorylation *in vitro* and is thus sufficient to be recognized by IκBα kinase(s). Due to the fact that the destruction box mediates proteolysis, it must also be sufficient for recognition by ubiquitin conjugating enzymes. In accordance with this, the critical determinants Ser32, Ser36, Lys21 and Lys22 and a recently identified ubiquitin ligase binding site (32) are contained within the destruction box. Furthermore, the sequence contains Tyr42, required for IκBα release from NF-κB by a different mechanism not requiring degradation (5).

Recently Brown *et al*. (29) reported that the C-terminal PEST sequence of IκBα cooperated with the M-terminal SRD to direct PMA/ionomycin-induced proteolysis of chimeric IκBα–Bcl-3 proteins, as well as chimeric glutathione S-transferase proteins. Under their experimental conditions the PEST sequence could be transferred to a heterologous protein containing the SRD sequence, leading to enhanced degradation. The requirement for the C-terminal PEST motif in IκBα for induced degradation has in fact been a controversial issue in a number of studies (8,10,11,24,25,33,34 and references therein). Our findings clearly show that the IκBα SRD can act independently of the PEST domain in IκBαΔC or when linked to Bcl-3, p50 or p53. The presence of the PEST domain was not absolutely required for degradation of the chimeric proteins, although it did enhance the efficiency of IκBα degradation.

Two alternative hypotheses might account for these observations. The negatively charged PEST sequence in IκBα increases the interaction strength towards NF-κB (26,27, see 28 for discussion). It is possible that IκBα–NF-κB complexes are the preferred substrate for the IκBα kinase *in vivo*. Activation of the kinase in response to TNF-α is transient, reaching a peak 5 min after stimulation and dissipating by 30 min (31). This may relate to more efficient degradation of IκBαΔC and of the chimera during long-term stimulation by OA compared with TNF-α or PMA stimulation, when the kinase activity is limiting. *In vitro* the kinase is maintained in the active state by the presence of OA in the reaction buffer (31). Under these conditions IκBα and the chimera containing the SRD were phosphorylated with comparable efficiency (Fig. 2). Preferential activity of the IκBα kinase toward preformed NF-κB–IκBα complexes is an attractive possibility and is consistent with rapid recovery of IκBα levels by *de novo* synthesis after cellular stimulation (35,36). Alternatively, it is feasible that in the context of native IκBα the PEST motif might contribute to a structural conformation that is favourable for recognition by the IκBα kinase or that assists in the transfer of ubiquitinated IκBα to the proteasome (37). We cannot rule out the possibility that Bcl-3, p50 and p53 each contain sequences that can substitute for the PEST sequence at a required step in the degradation pathway. The constructs tested by Brown *et al*. might lack such compensatory sequences (29).

Both the PEST motif and the sequence surrounding the serine residues in the SRD of IκBα are conserved in other members of the IκB family as well. In *Drosphila* Cactus the SRD, but not the PEST motif, is required for signal-induced degradation (38). In contrast, the PEST sequence in human IκBβ strongly promotes efficient inducible degradation (39 and references therein). Thus the structural differences between these related molecules may determine how far the SRD can direct inducible degradation by itself.

It is interesting to compare IκBα to cyclin E; the regulated degradation of cyclin E is under the control of a PEST-like element in the C-terminus of the protein. In this case the PEST motif and surrounding sequences function as a substrate for cyclin E/cdk2 autophosphorylation, triggering cyclin E dissociation and enhanced degradation (21,22). As has been shown for IκBα (28), free cyclin E is degraded by a mechanism that is not dependent on the PEST motif (21). Interestingly, phosphorylation of IκBα *in vitro* by both protein kinase A and protein kinase C can disrupt the IκBα–NF-κB complex (40,41); *in vitro* both kinases target Ser262, immediately adjacent to the IκBα PEST element (D.Krappmann and C.Scheidereit, unpublished observations).

It is possible that alternative inducer-specific pathways exist that are relevant to regulation of IκBα degradation, as is the case for tyrosine phosphatase inhibitors (5). Nevertheless, the data presented here clearly demonstrate that the N-terminal IκBα destruction box can act autonomously to direct degradation of heterologous proteins. Our inability to dissect induced phosphorylation from induced degradation suggests that the sequence requirements for both reactions are highly related, if not identical. The IκBα kinase has been purified as a multisubunit 700 kDa complex containing E3 (ubiquitin ligase) activity (17). It therefore appears likely that phosphorylation and ubiquitination of the SRD occurs in a concerted manner. A similar mechanism has recently been demonstrated for the yeast G1 cyclins Cln 1–3 (42 and references therein). Hyperphosphorylation of the C-terminal Cln destruction box is associated with their instability. Phosphorylation promotes binding by the Cdc53 protein, an E3 ubiquitin ligase with specificity for Cln proteins. Cdc53 in turn recruits the E2 ubiquitin ligase Cdc34, leading to ubiquitination of Cln (and Cdc53). The E3 enzyme specific for phosphorylated IκBα remains to be identified; E2 enzymes with broad specificity can support ubiquitination of IκBα (15,17).

To our knowledge no other protein has yet been shown to contain a transferable destruction box that is activated by extracellular signalling pathways. The inverse situation has been described for c-Jun, which contains a constitutive destruction box that is inactivated in response to Map kinase signalling (43,44).

Ligand binding by the yeast G protein receptor Ste2p induces its ubiquitination within the sequence SINNDAKKS, resulting in receptor endocytosis as opposed to degradation by the proteasome (45). A destruction box located in Rag-2 is regulated by phosphorylation and may be functionally related to those found in cell cycle-regulated proteins (46). Destruction boxes identified in mitotic cyclins and in G1 cyclins each function in a heterologous context (47–50, reviewed in 20); regulation can be at the level of substrate phosphorylation or E3 activation (20,38).

Each of these examples illustrates the fundamental role ubiquitin-mediated proteolysis plays in regulation of cell function and the validity of the approach we have chosen to investigate IκBα degradation. Identification of the specificity determinants governing IκBα destruction should aid in ongoing characterization of the enzymes involved and the reaction pathway used.

ACKNOWLEDGEMENTS

We thank Dr A.Israel for the gift of the IκBα Ala32/Ala36 double mutant. We gratefully acknowledge Erika Scharschmidt for dedicated technical assistance. This work was in part supported by a grant from the DFG (SFB 344) to C.S.

REFERENCES

1 Verma,I.M., Stevenson,J.K., Schwarz,E.M., Van Antwerp,D. and Miyamoto,S. (1995) *Genes Dev.*, **9**, 2723–2735.
2 Finco,T.S. and Baldwin,A.S. (1995) *Immunity*, **3**, 263–272.
3 Baeuerle,P.A. and Baltimore,D. (1996) *Cell*, **87**, 13–20.
4 Wulczyn,F.G., Krappmann,D. and Scheidereit,C. (1996) *J. Mol. Med.*, **74**, 749–769.
5 Imbert,V., Rupec,R.A., Livolsi,A., Pahl,H.L., Traenckner,E.B.-M., Mueller-Dieckmann,C., Farahifar,D., Rossi,B., Auberger,P., Baeuerle,P.A. and Peyron,J.-F. (1996) *Cell*, **86**, 787–798.
6 Hochstrasser,M. (1996) *Cell*, **84**, 813–815.
7 Brockman,J.A., Scherer,D.C., McKinsey,T.A., Hall,S.M., Qi,X., Lee,W.Y. and Ballard,D.W. (1995) *Mol. Cell. Biol.*, **15**, 2809–2818.
8 Brown,K., Gerstberger,S., Carlson,L., Franzoso,G. and Siebenlist,U. (1995) *Science*, **267**, 1485–1488.
9 Traenckner,E.B., Pahl,H.L., Henkel,T., Schmidt,K.N., Wilk,S. and Baeuerle,P.A. (1995) *EMBO J.* **14**, 2876–2883.
10 Whiteside,S.T., Ernst,M.K., LeBail,O., Laurent-Winter,C., Rice,N. and Israël,A. (1995) *Mol. Cell. Biol.*, **15**, 5339–5345.
11 Chen,Z., Hagler,J., Palombello,V.J., Melandri,F., Scherer,D., Ballard,D. and Maniatis,T. (1995) *Genes Dev.*, **9**, 1586–1597.
12 Scherer,D.C., Brockman,J.A., Chen,Z.J., Maniatis,T. and Ballard,D.W. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 11259–11263.
13 Baldi,L., Brown,K. and Siebenlist,U. (1996) *J. Biol. Chem.*, **271**, 376–379.
14 DiDonato,J., Mercurio,F., Rosette,C., Wu-Li,J., Suyang,H., Ghosh,S. and Karin,M. (1996) *Mol. Cell. Biol.*, **16**, 1295–1304.
15 Alkalay,I., Yaron,A., Hatzubai,A., Orian,A., Ciechanover,A. and Ben-Neriah,Y. (1995) *Proc. Natl. Acad. Sci USA*, **92**, 10599–10603.
16 Li,C.C., Dai,R.M. and Longo,D.L. (1995) *Biochem. Biophys. Res. Commun.*, **215**, 292–301.
17 Chen,Z.J., Parent,L. and Maniatis,T. (1996) *Cell*, **84**, 853–862.
18 Rogers,S., Wells,R. and Rechsteiner,M. (1986) *Science*, **234**, 364–368.
19 Smith,S.E., Koegl,M. and Jentsch,S. (1996) *Biol. Chem. Hoppe Seyler*, **377**, 437–446.
20 Hochstrasser,M. (1996) *Annu. Rev. Genet.*, **30**, 405–439.
21 Clurman,B.E., Sheaff,R.J., Thress,K., Groudine,M. and Roberts,J.M. (1996) *Genes Dev.*, **10**, 1979–1990.
22 Won,K.A. and Reed,S.I. (1996) *EMBO J.*, **15**, 4182–4193.
23 Sun,S.-C., Elwood,J. and Greene,W.C. (1996) *Mol. Cell. Biol.*, **16**, 1058–1065.
24 Aoki,T., Sano,Y., Yamamoto,T. and Inoue,J. (1996) *Oncogene*, **12**, 1159–1164.
25 Van Antwerp,D.J. and Verma,I.M. (1996) *Mol. Cell. Biol.*, **16**, 6037–6045.
26 Hatada,E.N., Naumann,M. and Scheidereit,C. (1993) *EMBO J.*, **12**, 2781–2788.
27 Ernst,M.K., Dunn,L.L. and Rice,N.R. (1995) *Mol. Cell. Biol.*, **15**, 872–882.
28 Krappmann,D., Wulczyn,F.G. and Scheidereit,C. (1996) *EMBO J.*, **15**, 6716–6726.
29 Brown,K., Franzoso,G., Baldi,L., Carlson,L., Mills,L., Lin,Y.-C., Gerstberger,S. and Siebenlist,U. (1997) *Mol. Cell. Biol.*, **17**, 3021–3027.
30 Naumann,M., Wulczyn,F.G. and Scheidereit,C. (1993) *EMBO J.*, **12**, 213–222.
31 Lee,F.S., Hagler,J., Chen,Z.J. and Maniatis,T. (1997) *Cell*, **88**, 213–222.
32 Yaron,A., Gonen,H., Alkalay,I., Hatzubai,A., Jung,S., Beyth,S., Mercurio,F., Manning,A.M., Ciechanover,A. and Ben-Neriah,Y. (1997) *EMBO J.*, **16**, 6486–6494.
33 Rodriguez,M., Michalopoulos,I., Arenzana-Seisdedos,F. and Hay,R. (1995) *Mol. Cell. Biol.*, **15**, 2413–2419.
34 McElhinny,J.A., Trushin,S.A., Bren,G.D., Chester,N. and Paya,C.V. (1996) *Mol. Cell. Biol.*, **16**, 899–906.
35 Sun,S.C., Ganchi,P.A., Ballard,D.W. and Greene,W.C. (1993) *Science*, **259**, 1912–1915.
36 Scott,M.L., Fujita,T., Liou,H.C., Nolan,G.P. and Baltimore,D. (1993) *Genes Dev.*, **7**, 1266–1276.
37 Kroll,M., Conconi,M., Desterro,M.J., Marin,A., Thomas,D., Friguet,B., Hay,R.T., Virelizier,J.L., Arenzana-Seisdedos,F. and Rodriguez,M.S. (1997) *Oncogene*, **15**, 1841–1850.
38 Bergmann,A., Stein,D., Geisler,R., Hagenmaier,S., Schmid,B., Fernandez,N., Schnell,B. and Nusslein-Volhard,C. (1996) *Mech. Dev.*, **60**, 109–123.
39 Hirano,F., Chung,M., Tanaka,H., Maruyama,N., Makino,I, Moore,D.D. and Scheidereit,C. (1998) *Mol. Cell. Biol.*, **18**, in press.
40 Shirakawa,F. and Mizel,S.B. (1989) *Mol. Cell. Biol.*, **9**, 2424–2430.
41 Ghosh,S. and Baltimore,D. (1990) *Nature*, **344**, 678–682.
42 Willems,A.R., Lanker,S., Patton,E.E., Craig,K.L., Nason,T.F., Mathias,N., Kobayashi,R., Wittenberg,C. and Tyers,M. (1996) *Cell*, **86**, 453–463.
43 Treier,M., Staszewski,L.M. and Bohmann,D. (1994) *Cell*, **78**, 787–798.
44 Musti,A.M., Treier,M. and Bohmann,D. (1997) *Science*, **275**, 400–402.
45 Hicke,L. and Riezman,H. (1996) *Cell*, **84**, 277–287.
46 Lin,W.C. and Desiderio,S. (1993) *Science* , **260**, 953–959.
47 Glotzer,M., Murray,A.W. and Kirschner,M.W. (1991) *Nature*, **349**, 132–138.
48 Yaglom,J., Linskens,M.H., Sadis,S., Rubin,D.M., Futcher,B. and Finley,D. (1995) *Mol. Cell. Biol.*, **15**, 731–741.
49 King,R.W., Glotzer,M. and Kirschner,M.W. (1996) *Mol. Biol. Cell*, **7**, 1343–1357.
50 Brandeis,M. and Hunt,T. (1996) *EMBO J.*, **15**, 5280–5289.